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| <b>(54) Title:</b> IN VITRO GENERATION OF HUMAN DENDRITIC CELLS AND USES THEREOF<br><br><b>(57) Abstract</b><br><br>A method is provided for generating human dendritic cells <i>in vitro</i> by treating CD34 <sup>+</sup> cells with tumor necrosis factor- $\alpha$ and interleukin-3 or with GM-CSF. The invention also includes cellular compositions of dendritic cells produced by this method. Dendritic cells of the invention can be used widely as components in many diagnostic and therapeutic systems, including improved mixed lymphocyte reactions for assaying tissue rejection, adoptive immunotherapy of cancer, adoptive immunotherapy of HIV and other viral infections, and SCID-hu mice for human antibody production. |           |   |

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## IN VITRO GENERATION OF HUMAN DENDRITIC CELLS AND USES THEREOF

The invention relates generally to an *in vitro* method of generating human dendritic cells, and, more specifically, to therapeutic and diagnostic uses of the generated cells.

Dendritic cells are a system of antigen-presenting cells that function to initiate several immune responses such as the sensitization of MHC-restricted T cells, the rejection of organ transplants, and the formation of T cell-dependent antibodies. Dendritic cells are found in many nonlymphoid tissues but can migrate *via* the afferent lymph or the blood stream to the T cell-dependent areas of lymphoid organs. They are found in the skin, where they are named Langerhans cells, and are also present in the mucosa. They represent the sentinels of the immune system within the peripheral tissues where they can acquire antigens.

As these cells express CD4 and can be infected *in vitro* by HIV, they are likely to present a port of entry of HIV virus *in vivo*: *e.g.* Knight et al., pp. 145 in Racz, et al., editors, "Accessory Cells in HIV and Other Retroviral Infections" (Karger, Basel, 1991); Ramsauer et al., pp. 155 in Racz, et al., editors (cited above). The isolation of human dendritic cells from peripheral blood has only recently been achieved and only small numbers of cells can be generated, *e.g.* Freudenthal et al., *Proc. Natl. Acad. Sci.*, Vol. 87, pp. 7698 (1990). The *in vitro* generation of large numbers of human dendritic cells would present an important advantage for priming *in vitro* human naive CD4 and CD8 T cells, for screening agents that may interfere with HIV infection, for generating primary and secondary *in vivo* response of human B cells in SCID-hu mice reconstituted with human T and B cells, and for constructing a more sensitive mixed-lymphocyte reaction assay.

A major impediment to transplantation of allogenic tissue and organs is graft rejection by the transplant recipient. The cell-mediated immune reaction of the recipient, or host, to the donor tissue plays an

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important role in the rejection process. The cell-mediated immune response has two important phases: (i) recognition, when host cells recognize the donor cell as foreign in the context of the major histocompatibility complex (MHC); and (ii) destruction, when the host cells respond by attacking the foreign cells. As part of the attacking process, a number of responder cells undergo proliferation and acquire cytotoxicity — that is, the ability to kill donor cells displaying the appropriate antigens. Thus, cell-mediated immunity can be described in terms of two measurable functions: proliferation, and cytotoxic activity — see Dubey et al., chapter 131 in Rose et al., Editors, "Manual of Clinical Laboratory Immunology", 3rd edition (American Society of Microbiology, Washington, D.C., 1986).

Development of cell culture techniques has led to the establishment of *in vitro* methods that mimic the *in vivo* immunization process, thus providing measures for the assessment of cell-mediated immunity *in vitro*. Of particular utility in regard to transplantation is the mixed lymphocyte response (MLR), or mixed lymphocyte culture. The MLR is a relatively simple assay, yet it exists in many variants. Typically, the assay consists of mixing responder lymphocytes in a suitable culture system with stimulator lymphocytes whose proliferation and/or transcription machinery has been disabled, *e.g.* by irradiation. After the cells have been cultured for several days, a number of different measurements can be made to quantify the degree of reactivity of the responder cells to the stimulator cells, *e.g.* uptake of tritiated thymidine, number of blast cells, number of dividing cells, cytokine production, and the like. Other variables in the assay include the source of the responder and stimulator cells, *e.g.* peripheral blood, spleen, lymph nodes, *etc.*; whether the responder cells are syngenic, allogenic, or xenogenic with respect to the stimulator cells; the method of disabling the stimulator cells, for example irradiation or treatment with a DNA synthesis inhibitor (*e.g.* mitomycin C) or the like.

A drawback of the MLR as a routine assay for cell-mediated immune reactivity is sensitivity. Frequently, it is difficult to obtain a strong effect in the MLR, whatever the particular read-out employed. It is believed that antigen-presenting cells in the stimulator population are

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responsible for stimulating the responder cells; however, in most tissue sources such cells are few in number and/or are of a type that stimulates inefficiently. The sensitivity, and hence the utility, of the MLR assay could be greatly enhanced by the availability of more potent stimulator cell populations. Dendritic cells could serve this function, since they are well known as potent antigen-presenting cells, *e.g.* Steinman, *Ann. Rev. Immunol.*, Vol. 9, pgs. 271-296 (1991). Unfortunately, it is presently very difficult to obtain them in quantities sufficient for routine MLRs, *e.g.* Steinman, et al., chapter 49 in Herzenberg et al., Editors, "Cellular Immunology" Vol. 2 (Blackwell Scientific Publications, Oxford, 1986).

#### Summary of the Invention

The invention is directed to a method for *in vitro* generation of human dendritic cells. The invention also includes isolated populations of human dendritic cells produced by the method of the invention and applications of the isolated cells, including an improved MLR assay that employs a pure population of dendritic cells as stimulator cells. The method of the invention includes the steps of culturing CD34<sup>+</sup> hematopoietic progenitor cells in the presence of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-3 (IL-3) or of granulocyte-macrophage colony stimulating factor (GM-CSF) to form CD1a<sup>+</sup> dendritic cells of the invention.

The invention therefore provides a process for the preparation of a cellular composition comprising human dendritic cells, comprising the steps of:

25       treating CD34<sup>+</sup> hematopoietic cells with TNF- $\alpha$  and IL-3 or with GM-CSF;

          and isolating the treated CD34<sup>+</sup> hematopoietic cells that express the CD1a antigen.

          Preferably the CD34<sup>+</sup> cells are treated with TNF- $\alpha$  in addition to  
30   GM-CSF.

The invention also provides CD34<sup>+</sup> hematopoietic cells that express the CD1a antigen following *in vitro* culture; and a cellular composition comprising human dendritic cells produced by the process defined above.

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Dendritic cells initiate immunological responses. The *in vitro* data reported herein for the dendritic cells according to the invention indicate that these cells are useful as laboratory tools and also may have utility in the *in vivo* treatment of various diseases by adoptive immunotherapy, including cancer and viral infections.

The invention further relates to the use of TNF- $\alpha$  together with IL-3 or of GM-CSF to prepare a dendritic cell line or to induce dendritic cells; to the use of dendritic cells to generate CD4<sup>+</sup> helper T cells; to the use of CD4<sup>+</sup> helper T cells in adoptive immunotherapy; to the use of SCID-hu mice for human antibody production; and to the use of dendritic cells to generate cancer-specific and virus-specific CD8<sup>+</sup> cytotoxic T cells, especially to the use of dendritic cells to generate CD8<sup>+</sup> cytotoxic T cells specific against the AIDS virus.

A further feature of the invention comprises a mixed lymphocyte reaction comprising the steps of:

- providing a sample of responder cells;
- providing a sample of inactivated stimulator cells such that the stimulator cells are allogenic with respect to the responder cells and such that the stimulator cells consist of dendritic cells produced by the process comprising the steps of (a) treating CD34<sup>+</sup> hematopoietic cells with TNF- $\alpha$  and IL-3 or with GM-CSF, and (b) isolating treated CD34<sup>+</sup> hematopoietic cells that express the CD1a antigen;
- co-culturing the responder cells and the inactivated stimulator cells; and
- measuring a response of the responder cells.

#### Brief Description of the Figures

Figure 1 illustrates flow cytometry data showing the partial co-expression of CD14 by CD1a<sup>+</sup> cells.

Figure 2 illustrates data concerning the growth kinetics of CD1a<sup>+</sup> cells under various medium conditions.

Figure 3 illustrates data of CD4<sup>+</sup> cell proliferation after stimulation by dendritic cells of the invention.

Figure 4 illustrates data showing the effect of CD1a<sup>+</sup> cells in stimulation of allogenic CD4<sup>+</sup> T cell proliferation.

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Figure 5 illustrates data of CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation after stimulation by dendritic cells of the invention.

### **DETAILED DESCRIPTION OF THE INVENTION**

An important aspect of the invention is the generation of dendritic cells from CD34<sup>+</sup> hematopoietic cells. CD34<sup>+</sup> hematopoietic progenitor cells can be obtained from a variety of tissue sources, *e.g.* bone marrow, but are preferably obtained from umbilical cord blood samples as follows: Light-density mononuclear cells from the samples are isolated by Ficoll-Hypaque gradient separation ( $d = 1.077$  g/mL) and are depleted of adherent cells, *e.g.* by overnight incubation at 37°C in RPMI 1640 medium supplemented with 1% w/v tissue-culture grade bovine serum albumin. Preferably, cells bearing CD34 antigen are isolated from non-adherent mononuclear fractions through positive selection by indirect immune panning using anti-CD34 monoclonal antibody, *e.g.* Imu-133.3 available from Immunotech (Marseille, France), anti-My 10 available from Becton Dickinson (Mountain View, California), or the like. Panning flasks are prepared as follows: sheep Fab antimouse IgG at a concentration of 25 µg/mL in Tris buffer (0.05 mol/L, pH 9.4) is distributed (10 mL) in 75-cm<sup>2</sup> tissue culture flasks for overnight coating at 4°C. Separately, the light-density mononuclear cells (depleted of adherent cells as discussed above) are incubated one hour at 4°C with 5 µg/mL anti-CD34 antibody at 10<sup>7</sup> cells/mL in RPMI 1640 supplemented with 2% heat-inactivated pooled human AB serum (HABS). Afterwards, cells are washed in cold medium containing 2% HABS, and 10 mL containing about 5 x 10<sup>7</sup> cells are distributed to the flasks previously coated with sheep antimouse IgG, as described above. Following a two-hour incubation at 4°C, non-adherent cells in suspension (*i.e.* the CD34-depleted fraction) are harvested by gentle pipetting and rinsing several times with medium. The adherent "panned" cells (*i.e.* the CD34-rich fraction) are then recovered by vigorous pipetting.

The dendritic cells are obtained from the CD34<sup>+</sup> cells by culturing these in medium containing GM-CSF or TNF-α and IL-3. Preferably, the dendritic cells are obtained from the CD34<sup>+</sup> cells by culturing these in medium containing TNF-α as well as GM-CSF. TNF-α, GM-CSF, and

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IL-3 suitable for use in the invention are commercially available, *e.g.* from Genzyme Corp. (Cambridge, MA), or can be produced by recombinant expression systems: *e.g.* as taught by Clark et al. in U.S. patent 4,959,455 (IL-3); Clark et al. in PCT application No. EP85/00326 (publ. no. WO86/00639) (GM-CSF); and Mark et al. in U.S. patent 4,677,063 (TNF- $\alpha$ ). Preferably, IL-3 and GM-CSF are used at saturating concentration; that is, they are used at a concentration at which all the IL-3 and GM-CSF receptors on the CD34<sup>+</sup> cells are occupied by biologically active IL-3 and GM-CSF molecules. Of course, the actual concentration may depend on the quality of IL-3 and GM-CSF used. Preferably, human IL-3 having a specific activity of at least  $5 \times 10^6$  U/mg is employed, wherein a unit of activity corresponds to the half-maximum proliferative activity as determined by <sup>3</sup>H-thymidine uptake by human bone marrow cells in liquid cultures. In the culture systems described below, saturating concentration was 10 ng/mL (or 50 U/mL). Preferably, human GM-CSF having a specific activity of at least  $2 \times 10^6$  U/mg is employed, wherein a unit of activity is as defined for IL-3 above. In the culture systems described below, saturating concentration was 100 ng/mL (or 200 U/mL). Preferably, TNF- $\alpha$  is used at a concentration in the range of 2 to 3 ng/mL or 40-60 U/mL, most preferably at a concentration of about 2.5 ng/mL or 50 U/mL. Units of TNF- $\alpha$  are defined by Carswell et al., *Proc. Natl. Acad. Sci.*, Vol. 72, pg. 3666 (1975), and by Aggarwal et al., *J. Biol. Chem.*, Vol. 260, pg. 2345 (1985). The cells can be co-cultured in standard tissue culture medium with standard additives, such as RPMI 1640 supplemented with 10% (v/v) heat inactivated fetal bovine serum, 10 mM Hepes, 2 mM L-glutamine,  $5 \times 10^{-5}$  M 2-mercaptoethanol, penicillin (100 U/mL) and streptomycin (100 mg/mL). Preferably, the CD34<sup>+</sup> cells are cultured in the presence of the cytokines for from 8 to 12 days.

Dendritic cells that form in the culture are isolated by panning as described above, with the exception that anti-CD1a and/or anti-CD14 antibodies are employed (both antibodies being commercially available, *e.g.* from Becton-Dickinson).

The MLR method of the invention comprises the following steps:  
(1) providing a sample of responder cells; (2) providing a sample of



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inactivated stimulator cells such that the stimulator cells are allogenic with respect to the responder cells and such that the stimulator cells consist of dendritic cells produced by the process comprising the steps of (a) treating CD34<sup>+</sup> hematopoietic cells with TNF- $\alpha$  and IL-3 or with GM-CSF, and (b) isolating treated CD34<sup>+</sup> hematopoietic cells that express the CD1a antigen; (3) co-culturing the responder cells and the inactivated stimulator cells; and (4) measuring a response of the responder cells.

Preferably, the sample of responder cells consists of CD4<sup>+</sup> T cells from the peripheral blood of a patient who is to be the recipient of a transplant. Obtaining T cell populations employs techniques well known in the art which are fully described by DiSabato et al., eds., in *Meth. in Enzymol.*, Vol. 108 (1984). For example, the CD4<sup>+</sup> T cells can be isolated as follows: first mononuclear cells are isolated from the peripheral blood and depleted of adherent cells; CD4<sup>+</sup> T cells are then purified by depleting other cell types, for example by immunomagnetic depletion (e.g. with Dynabeads, Dynal, Oslo, Norway), or the like, using a cocktail of commercially available monoclonal antibodies, e.g. anti-CD14, anti-CD16, anti-CD20, anti-CD8, anti-CD40 (available from Becton-Dickinson and/or Ortho Diagnostic Systems, New Jersey). CD4<sup>+</sup> populations having higher than 95% purity are typically achieved after two rounds of immunomagnetic depletion.

Stimulator cells of the invention are dendritic cells derived from CD34<sup>+</sup> hematopoietic progenitors cells obtained from a different person from that from whom the responder cells are taken; that is, the stimulator cells are allogenic with respect to the responder cells. These cells are obtained as described above.

The stimulator cells of the invention are inactivated so that they can still carry out their stimulatory function but are inhibited from any other function that could obscure the response measured from the responder cells. Thus, the nature of the inactivation depends somewhat on the "read-out" of the assay. Preferably, the read-out, or response measured in the responder cells, is cellular proliferation. Other read-outs could also include such phenomena as cytokine production, cytolytic ability, and the like. Preferably, the stimulator cells are treated

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so that they are incapable of replication, but their antigen-processing machinery remains functional. This is conveniently accomplished by irradiating the cells, *e.g.* with about 1500 to 5000 R (gamma or X-radiation), preferably 3000 to 4000 R, before mixing with the responder cells.

Preferably, proliferation of the responder cells is determined by the uptake of tritiated thymidine using standard protocols. For example, from  $10$  to  $2.5 \times 10^4$  stimulator cells are added to  $2.4 \times 10^4$  allogenic CD4<sup>+</sup> T cells in 96-well round-bottom tissue-culture plates and are incubated for 4 days in the medium described above. After incubation, the cells are pulsed with 1  $\mu$ Ci of tritiated thymidine for 6 hours, and then they are harvested and measured for tritiated thymidine uptake, *e.g.* by scintillation counting.

## EXPERIMENTAL

The following examples serve to illustrate the present invention. Cell lines, reagents and their concentrations, temperatures, and the values of other variables are only to exemplify the application of the present invention and are not to be considered limitations thereof.

### Example 1

#### Generation of Human Dendritic Cells

After 12 days of culture in GM-CSF and TNF- $\alpha$ , cells generated from CD34<sup>+</sup> cord blood hematopoietic precursor cells were processed for two-color fluorescence measurement. Briefly, cells were sequentially incubated with unconjugated monoclonal antibodies, phycoerythrin-conjugated (PE-conjugated) anti-mouse immunoglobulin, normal mouse serum, and monoclonal antibodies OKT6 (anti-CD1a from Ortho) or Leu-M3 (anti-CD14 from Becton-Dickinson), the monoclonal antibodies being directly labelled with fluorescein isothiocyanate (FITC). As shown in Figure 1, culturing CD34<sup>+</sup> cells for 12 days in the presence of GM-CSF and TNF- $\alpha$  allows the appearance of CD14<sup>+</sup> monocytic cells co-expressing the CD1a antigen. In addition, a population of CD14<sup>-</sup>CD1a<sup>+</sup> cells was consistently observed which represented

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30-90% of the total CD1a population (range from 5 experiments). Within the monocytic lineage, expression of CD1a antigen is restricted to Langerhans cells. Figure 1A shows two-color fluorescence intensity from IgG<sub>1</sub>-FITC isotype control versus IgG<sub>2a</sub>-PE isotype control. Figure 1B shows two-color fluorescence intensity from OKT6-FITC (proportional to CD1a expression) versus Leu-M3-PE (proportional to CD14 expression). As shown in Figure 2, CD1a<sup>+</sup> cells were not detected at the onset of the culture and the CD1a antigen could first be observed after 4 days of culture in the presence of TNF- $\alpha$ , and expression increased until day 20. Whereas less than 5% of CD1a<sup>+</sup> cells were observed in the presence of IL-3, 5-15% CD1a<sup>+</sup> cells were detected in GM-CSF. A large proportion of CD1a<sup>+</sup> cells were detected in IL-3 and TNF- $\alpha$  (10-25%) and mainly in GM-CSF and TNF- $\alpha$  (20-60%) (range of 5 experiments after 12 days of culture). In terms of growth efficiency, IL-3 plus TNF- $\alpha$  is 2-3 times more potent than IL-3 alone, and GM-CSF plus TNF- $\alpha$  is 3-4 times more potent than GM-CSF alone (Figure 2). Thus, starting from  $10^5$  CD34<sup>+</sup> cells, after 12 days in culture, GM-CSF allowed the generation of  $1-3 \times 10^6$  CD1a<sup>+</sup> cells, whereas in IL-3 plus TNF- $\alpha$  and GM-CSF plus TNF- $\alpha$  cultures of  $2-3 \times 10^6$  cells were recovered. As GM-CSF plus TNF- $\alpha$  appeared to be the most potent combination of factors for generating CD1a<sup>+</sup> cells, all the cultures for the characterization of those cells contained GM-CSF plus TNF- $\alpha$ : Figure 2 shows the expansion of  $10^5$  CD34<sup>+</sup> cells (i) in the presence of IL-3 (solid triangles), (ii) IL-3 and TNF- $\alpha$  (open triangles), (iii) GM-CSF (solid squares), and (iv) GM-CSF and TNF- $\alpha$  (open squares). Solid lines indicate total cell numbers in the three experiments, and dashed lines indicated expression of CD1a antigen. Figures 2A and 2B represent data from two separate sets of experiments.

The morphology of the CD1a<sup>+</sup> cells generated by the method of the invention was studied using both light and electron microscopy. Adherent cells observed in GM-CSF alone display the classical aspect of regularly shaped macrophages, whereas those obtained in the presence of GM-CSF plus TNF- $\alpha$  have a typical aspect of dendritic cells with highly ramified dendrites, lobulated nucleus, and a villous surface with dendritic projections. Some of the CD1a<sup>+</sup> cells (about 1 in 5)

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possess organelles with double membrane joining, recalling the structure of Birbeck granules.

The phenotype of CD1a<sup>+</sup> cells generated after 12 days of culture in the presence of GM-CSF and TNF- $\alpha$  was determined by two-color  
5 fluorescence analysis. Depending on the experiment, the percentage of CD1a<sup>+</sup> cells co-expressing CD14 varied between 10 and 70%. The results presented in Table I below were obtained from experiments in which less than 15% of CD1a<sup>+</sup> cells co-expressed CD14; thus, both CD1a<sup>+</sup> cells and CD1a<sup>-</sup>CD14<sup>+</sup> cells were characterized. CD1a<sup>+</sup> cells  
10 co-expressed CD1c, CD4, and CD40, but did not express CD1b. In contrast, CD1a<sup>-</sup>CD14<sup>+</sup> cells did not express CD1c and only weakly expressed CD4 and CD40. Both CD1a<sup>+</sup> and CD14<sup>+</sup> cells were found to bear Fc $\gamma$ RII (CD32), Fc $\gamma$ RIII (CD16), and CR3 (CD11b), whereas only CD1a<sup>-</sup>CD14<sup>+</sup> cells expressed Fc $\gamma$ RI (CD64) and CR1 (CD35). CD1a<sup>+</sup>  
15 and CD1a<sup>-</sup>CD14<sup>+</sup> cells expressed both LFA1 $\alpha$  (CD11a) and LFA1 $\beta$  (CD18), but CD1a<sup>+</sup> cells exhibited higher levels of ICAM1 (CD54) than CD1a<sup>+</sup>CD14<sup>+</sup> cells. CD1a<sup>+</sup> cells expressed very high levels of HLA-DR (5-10 times more than CD14<sup>+</sup> cells). In contrast, CD1a<sup>-</sup>CD14<sup>+</sup> cells expressed high levels of HLA-DQ<sup>+</sup>.

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**Table I****Phenotype of Dendritic Cells of the Invention**

| Antigen | Antibody                       | Reactivity with<br>CD1a cells | Reactivity with<br>CD14 cells | Source of<br>Antibody (*) |
|---------|--------------------------------|-------------------------------|-------------------------------|---------------------------|
| CD1a    | OKT6, IOT6a,<br>T6, DMC1, L544 | ++                            | -                             | Ort, Hy8,<br>Imu, Cou,    |
| CD1b    | IOT6b                          | -                             | -                             | Imu                       |
| CD1c    | IOT6c                          | +                             | -                             | Imu                       |
| CD14    | Leu-M3                         | +/-                           | +++                           | BD                        |
| CD4     | IOT4                           | ++                            | +/-                           | Imu                       |
| CD40    | Mab 89                         | ++                            | +/-                           | EP                        |
| CD16    | Leu11b                         | +                             | +                             | BD                        |
| CD32    | 2E1                            | +/-                           | +/-                           | Imu                       |
| CD64    | 197                            | -                             | +                             | Med                       |
| CD21    | CR2                            | -                             | -                             | BD                        |
| CD11b   | IOM1                           | ++                            | ++                            | Imu                       |
| CD35    | IOT17                          | -                             | ++                            | Imu                       |
| CD54    | 84H10                          | +++                           | +                             | Imu                       |
| CD11a   | SPVL7                          | ++                            | ++                            | Hy2                       |
| CD18    | BL5                            | ++                            | ++                            | Imu                       |
| HLA-DR  | L243                           | ++++                          | ++                            | BD                        |
| HLA-DQ  | SPVL3                          | +++                           | +                             | Imu                       |

\*Antibodies were obtained from sources as follows: Ort = Ortho Diagnostic Systems; Imu = Immunotech; Cou = Coulter (Hialeah, FL); BD = Becton-Dickinson; Med = Medarex Inc. (Lebanon, NH); Hy2 = Hybridoma, Vol. 2, pg. 423 (1983); Hy8 = Hybridoma, Vol. 8, pg. 199 (1989); EP=Eur. Pat. Appl. 89 403 491.7

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Example 2Proliferation of CD4<sup>+</sup> T Cells after Stimulation by Dendritic Cells

CD34<sup>+</sup> cells were cultured for 12 hours in accordance with the invention, after which they were irradiated with 4000 Rads to form stimulator cells for the experiment. From 10 to  $(2.4 \times 10^4)$  stimulator cells were seeded for  $2.5 \times 10^4$  resting CD4<sup>+</sup> T cells or other responder cells in round-bottomed microtest tissue culture plates in medium supplemented with 10% human AB<sup>+</sup> serum, as described below.

Dendritic cells of the invention were assayed for their capacity to induce resting allogenic CD4<sup>+</sup> T cells (as responder cells) to proliferate. As shown in Figure 3A, cells cultured in the presence of IL-3 alone (solid triangles) induced marginal allogenic CD4<sup>+</sup> T cell proliferation. In contrast, cells cultured in the presence of GM-CSF alone (solid squares), in the presence of IL-3 plus TNF- $\alpha$  (open triangles), and in the presence of GM-CSF plus TNF- $\alpha$  (open squares), induced a strong proliferation of allogenic CD4<sup>+</sup> T cells. Depending on culture conditions of the CD34<sup>+</sup> progenitor cells, the optimal proliferation of the CD34<sup>+</sup> T cells was observed for different values of the ratio (stimulator cells)/(allogenic CD34<sup>+</sup> T cells). In comparison with control values (CD34<sup>+</sup> T cells without stimulator cells), a 50-fold enhancement of tritiated thymidine uptake by CD4<sup>+</sup> T cells was observed at a ratio of 1:3.8 (range 1:3 to 1:25), 1:12.5 (range 1:10 to 1:35), and 1:360 (range 1:100 to 1:400) for cells cultured in the presence of GM-CSF alone, IL-3 plus TNF- $\alpha$ , and GM-CSF plus TNF- $\alpha$  respectively (ranges from 5 experiments).

In the Experiment illustrated in Figure 3B, the CD34<sup>+</sup> cells were cultured in the presence of GM-CSF and TNF- $\alpha$  for all experiments and the responder cells were adult peripheral blood (open squares), cord blood (open and solid triangles), and syngenic cord blood CD4<sup>+</sup> T cells (solid squares). Figure 3B shows that allogenic CD4<sup>+</sup> T cells derived either from cord blood or from adult peripheral blood were equally stimulated, and that syngenic CD4<sup>+</sup> T cells were stimulated to a much lower extent (response 20-fold weaker than allogenic cells).

When, after 12 days of culture in the presence of GM-CSF and TNF- $\alpha$ , CD1a<sup>+</sup> cells were removed by immunomagnetic depletion

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(Figure 4), a strong loss of induction capacity was observed. A 50-fold enhancement of CD4<sup>+</sup> T cell proliferation was observed for a ratio (stimulator cells)/(allogenic CD4<sup>+</sup> T cells) of 1:200 (range of 3 experiments 1:200 to 1:400) and 1:8 (range of 3 experiments 1:8 to 1:40) before and after CD1a<sup>+</sup> cells were depleted, respectively. After irradiation, the following were used as stimulator cells: total population (solid squares), population from which CD1a<sup>+</sup> cells had been removed by depletion (open squares), and control population from which depletion was carried out with an anti-IgG<sub>1</sub> isotype antibody (open triangles).

### Example 3

#### Generation of Cancer-specific CD8 Cytotoxic

#### T Cells for Adoptive Immunotherapy

CD34<sup>+</sup> cells are isolated from the peripheral blood of a cancer patient and grown in the presence of GM-CSF and TNF- $\alpha$ , as described above. Peripheral blood CD8 cells are cryopreserved. Once dendritic cells are generated, CD8 T cells are thawed and mixed with the patient's cancer cells. After sensitization, CD8 T cells are expanded in the presence of IL-2, e.g. as described by Rosenberg, U.S. patent 4,690,915. Total cells are reinfused into the patient provided that they display tumor-specific cytotoxic activity. The tumor cells may be replaced by specific tumor antigens, e.g. van der Bruggen et al., *Science*, Vol. 254, pp. 1643 (1991).

Dendritic cells generated in the presence of GM-CSF and TNF- $\alpha$  are strong stimulators of resting allogenic CD8<sup>+</sup> T cell proliferation

Dendritic cells generated from CD34<sup>+</sup> cord blood progenitors were cultured for 12 days and irradiated (4000 Rads), and then were used as stimulator cells for resting CD4<sup>+</sup> and CD8<sup>+</sup> T cells. From 10 to  $2.5 \times 10^3$  stimulator cells were seeded for  $2 \times 10^4$  resting T cells, in round-bottomed microtest tissue-culture plates, in medium supplemented with 10% human AB<sup>+</sup> serum, with or without 20 U/ml IL-2. After 5 days' incubation, cells were pulsed with 1  $\mu$ Ci of <sup>3</sup>H-thymidine for 8 hours, harvested and counted. Tests were carried out in triplicate and results were expressed as mean counts per minute.

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The addition of IL-2 to the medium in which the CD4<sup>+</sup> and CD8<sup>+</sup> T cells were cultured indeed stimulated the proliferation of the CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as shown by their <sup>3</sup>H-thymidine uptake. As shown in Figure 5, CD8<sup>+</sup> cells cultured in the presence of medium alone (open squares) showed very little proliferation whereas CD8<sup>+</sup> cells cultured in the presence of IL-2 (solid squares) showed much more proliferation; moreover, CD4<sup>+</sup> cells cultured in the presence of medium alone (open circles) showed little proliferation whereas CD4<sup>+</sup> cells cultured in the presence of IL-2 (solid circles) showed considerably more proliferation.

10

#### Example 4

##### Generation of Primary and Secondary Antibody Responses in SCID-hu Mice

SCID-hu mice have allowed secondary responses to recall antigens but have not permitted the establishment of primary responses: e.g. Moller, *The SCID-hu Mouse*, Vol. 124 (Munksgaard, Copenhagen, 1991); Duchosal et al., *Nature*, Vol. 355, pp. 258 (1992); and Mosier et al., *Curr. Top. Microbiol. Immunol.*, Vol. 152, pp. 195 (1989). It is believed that this is due to a lack of reconstitution of the dendritic cell pool. As antigen-pulsed dendritic cells can efficiently induce an antibody response *in vivo*, e.g. Sornasse et al., *J. Exp. Med.*, Vol. 175, pp. 15 (1992), SCID-hu mice are reconstituted with the dendritic cells generated *in vitro* from the CD34 cells of the donor of human cells or from another donor sharing a compatible MHC. Dendritic cells are pulsed with the appropriate antigen prior to injection. Once the mouse displays antibody to the antigen, B cells are isolated from blood and other reconstituted organs and immortalized, e.g. by culturing in the CD40 system in the presence of Epstein-Barr virus as taught by Banchereau et al., *Science*, Vol. 251, pp. 70 (1991).

30

#### Example 5

##### Treatment of AIDS by Adoptive Immunotherapy with Dendritic Cells Generated *In Vitro*

Dendritic cells are infected by HIV *in vitro* and HIV is found budding from the surface of the cells after 3-5 days in culture with HIV: e.g. Patterson, *J. Gen. Virol.*, Vol. 68, pgs. 1177-1181 (1987); Macatonia



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- et al., *Immunology*, Vol. 71, pgs. 38-45 (1989); and Knight et al., *Immunol. Lett.*, Vol. 19, pgs. 177-182 (1988). Evidence for *in vivo* infection of dendritic cells by HIV comes from the description of infection of Langerhans cells of the skin and reduction in the amount of MHC class II molecules in cells of the skin: e.g. Tschachler et al., *J. Invest. Dermatol.*, Vol. 88, pgs. 233-237 (1987); and Belsito et al., *N. Engl. J. Med.*, Vol. 310, pgs. 1279-1282 (1984). Furthermore, 3-21% of blood dendritic cells from HIV patients contain HIV, with a level of infection two orders of magnitude greater than that seen in other cell types:
- 5      Macatonia et al., *Immunology*, Vol. 71, pgs. 38-45 (1990). The infection of dendritic cells by the virus blocks their ability to present antigen to T cells, and thus inhibits the recruitment/proliferation of the T cells which is necessary for a successful immune response.
- 10      CD34<sup>+</sup> cells of HIV patients are used to generate dendritic cells in accordance with the invention. The dendritic cells are then incubated with selected antigens and reinfused into the HIV patient.
- 15

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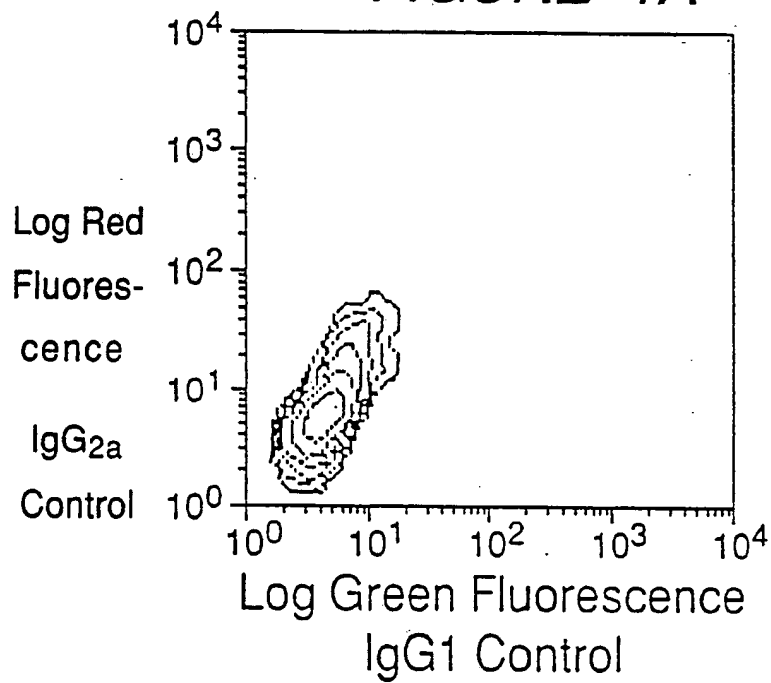
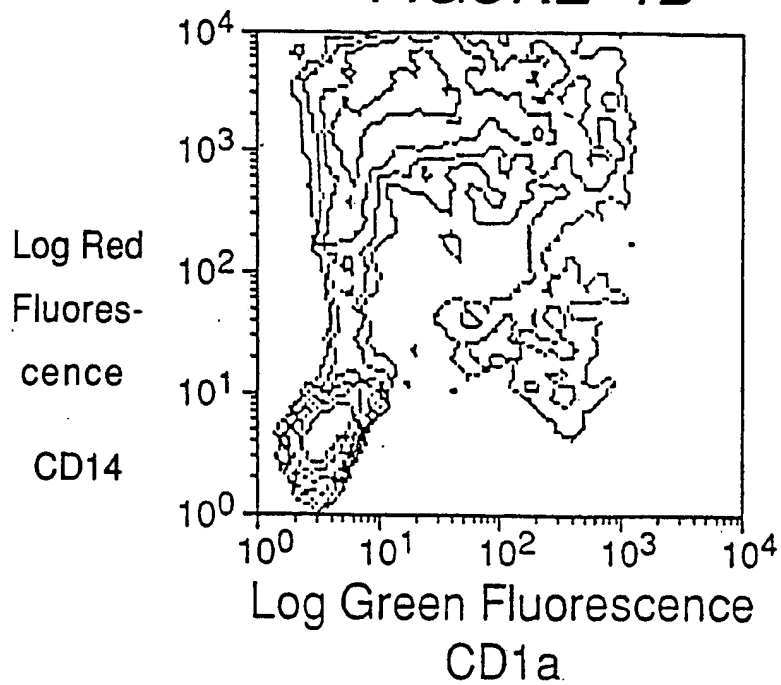
CLAIMS:

1. A process for the preparation of a cellular composition comprising human dendritic cells, comprising the steps of:  
treating CD34<sup>+</sup> hematopoietic cells with TNF- $\alpha$  and IL-3 or with  
5 GM-CSF;  
and isolating the treated CD34<sup>+</sup> hematopoietic cells that express the CD1a antigen.
2. A process as claimed in claim 1 wherein said CD34<sup>+</sup> cells are treated with TNF- $\alpha$  in addition to GM-CSF.
- 10 3. CD34<sup>+</sup> hematopoietic cells that express the CD1a antigen following *in vitro* culture.
4. A cellular composition comprising human dendritic cells produced by the process comprising the steps of:  
treating CD34<sup>+</sup> hematopoietic cells with TNF- $\alpha$  and IL-3 or with  
15 GM-CSF;  
and isolating the treated CD34<sup>+</sup> hematopoietic cells that express the CD1a antigen.
5. The cellular composition of claim 4 wherein said CD34<sup>+</sup> cells are treated with TNF- $\alpha$  in addition to GM-CSF.
- 20 6. Use of TNF- $\alpha$  together with IL-3 or of GM-CSF to prepare a dendritic cell line.
7. Use of TNF- $\alpha$  together with IL-3 or of GM-CSF to induce dendritic cells.
8. Use of dendritic cells to generate CD4<sup>+</sup> helper T cells.
- 25 9. Use of CD4<sup>+</sup> helper T cells in adoptive immunotherapy.
10. Use of dendritic cells to generate cancer-specific and virus-specific CD8<sup>+</sup> cytotoxic T cells.

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11. Use of dendritic cells to generate CD8<sup>+</sup> cytotoxic T cells specific against the AIDS virus.
12. A mixed lymphocyte reaction comprising the steps of:  
providing a sample of responder cells;  
5 providing a sample of inactivated stimulator cells such that the stimulator cells are allogenic with respect to the responder cells and such that the stimulator cells consist of dendritic cells produced by the process comprising the steps of (a) treating CD34<sup>+</sup> hematopoietic cells with TNF- $\alpha$  and IL-3 or with GM-CSF, and (b) isolating treated CD34<sup>+</sup>  
10 hematopoietic cells that express the CD1a antigen;  
co-culturing the responder cells and the inactivated stimulator cells;  
and  
measuring a response of the responder cells.
13. The method of claim 12 wherein said CD34<sup>+</sup> cells are treated  
15 with TNF- $\alpha$  and GM-CSF.
14. The method of claim 12 wherein said response of said responder cells is proliferation.
15. The method of claim 12 wherein IL-3 and GM-CSF are used at saturating concentration.
- 20 16. The method of claim 12 wherein TNF- $\alpha$  is used at a concentration in the range of 2 to 3 ng/mL or 40-60 U/mL.
17. The method of claim 12 wherein TNF- $\alpha$  is used at a concentration of about 2.5 ng/mL or 50 U/mL.
18. A method of treating a patient suffering from cancer, which  
25 comprises administering to said patient cells as claimed in claim 3.
19. A method of treating a patient suffering a viral infection, which comprises administering to said patient cells as claimed in claim 3.
20. A method as claimed in claim 18 or claim 19 wherein the cells are derived from the patient.

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**FIGURE 1A****FIGURE 1B**

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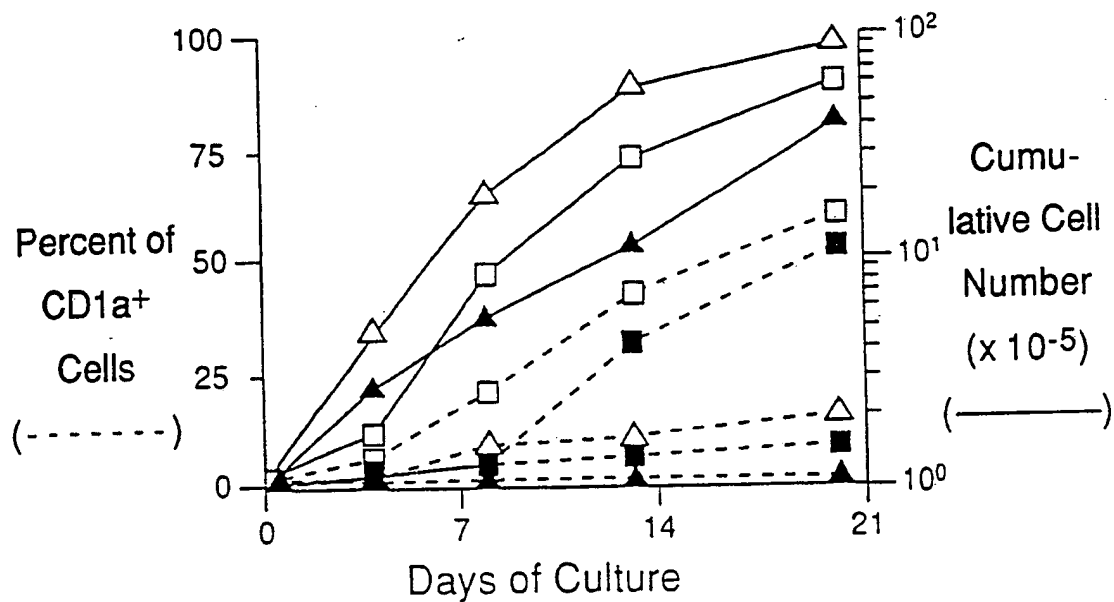
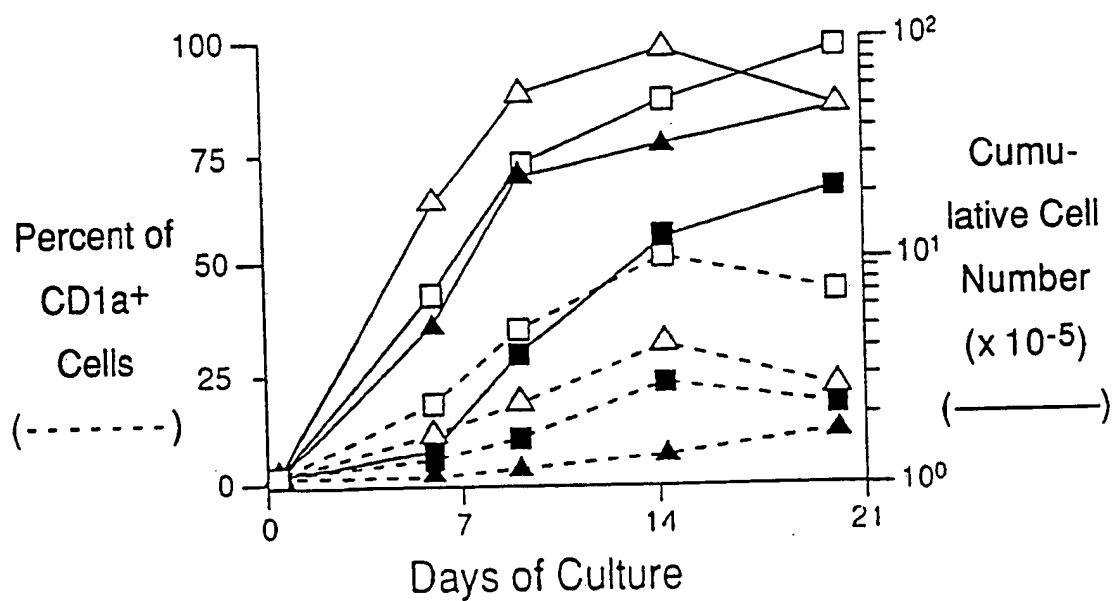
**FIGURE 2A****FIGURE 2B**

FIGURE 3A

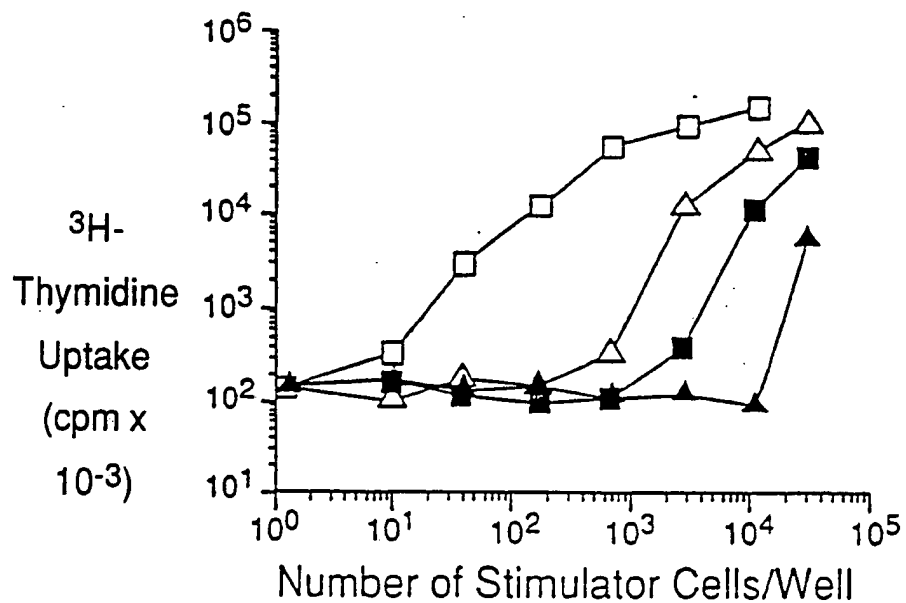
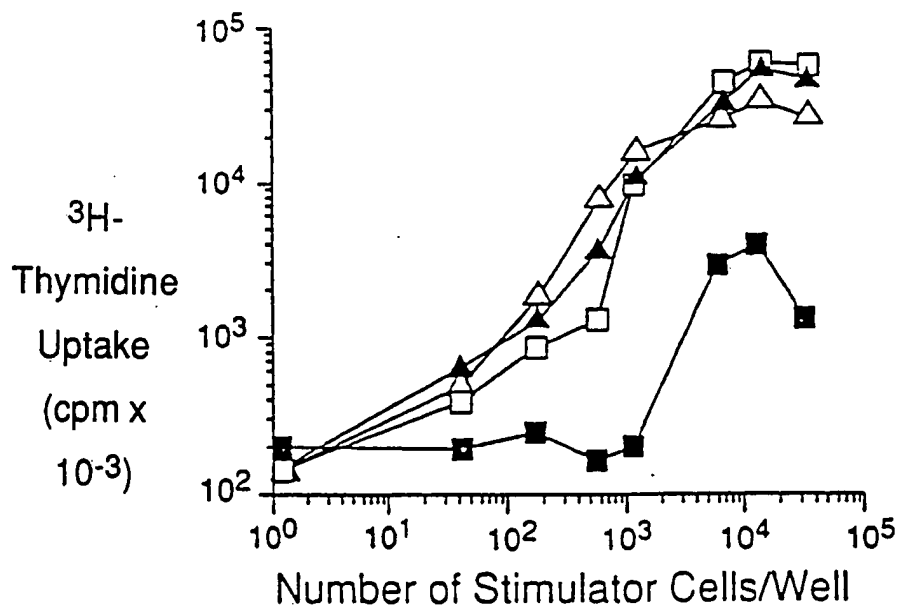
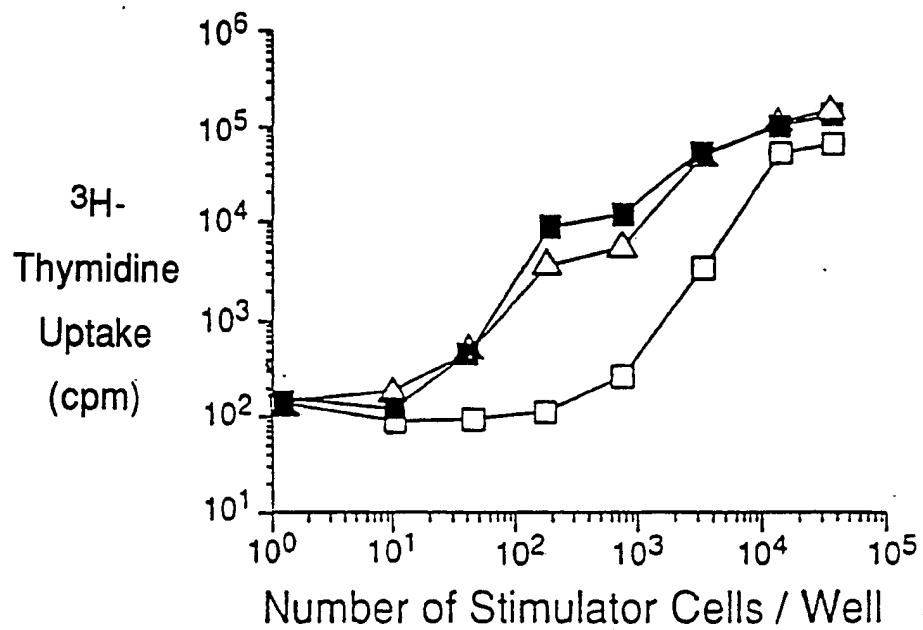


FIGURE 3B



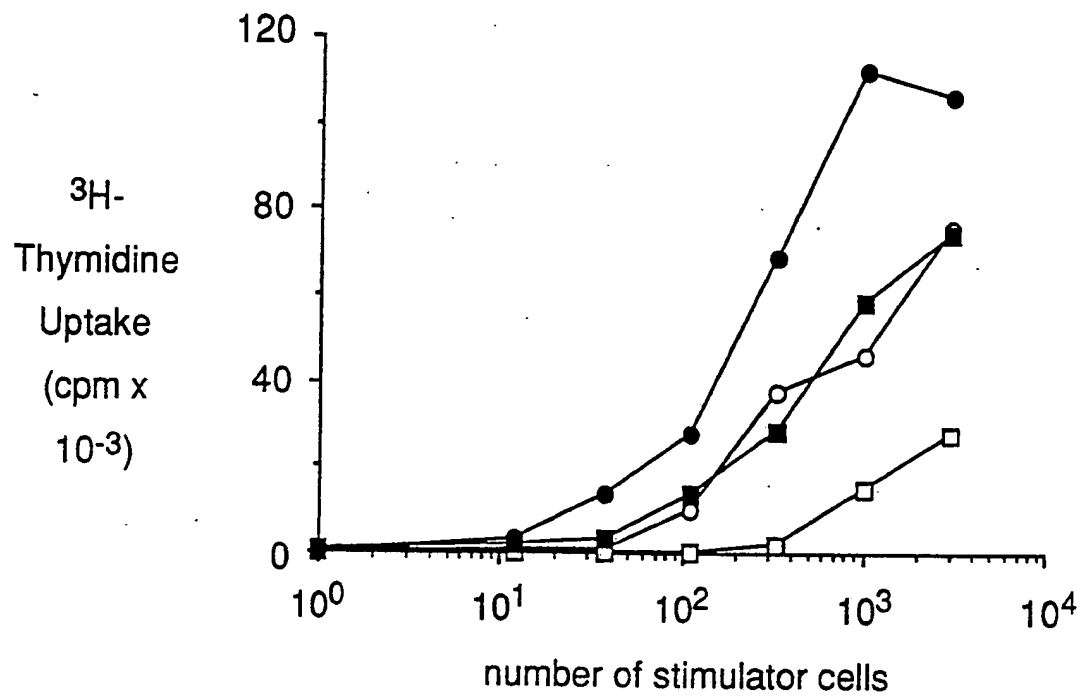
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FIGURE 4



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FIGURE 5



- CD4 + medium
- CD4 + IL-2
- CD8 + medium
- CD8 + IL-2



|   |  |                                     |
|---|--|-------------------------------------|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>   |  |                                     |
| According to International Patent Classification (IPC) or to both National Classification and IPC<br>Int.Cl. 5 C12N5/08   |  |                                     |
| <b>II. FIELDS SEARCHED</b>  |  |                                     |
| Minimum Documentation Searched <sup>7</sup>   |  |                                     |
| Classification System   | Classification Symbols   |                                     |
| Int.Cl. 5   | C12N ; C07K  |                                     |
| Documentation Searched other than Minimum Documentation<br>to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>   |  |                                     |
|   |  |                                     |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>   |  |                                     |
| Category <sup>9</sup>   | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>   | Relevant to Claim No. <sup>13</sup> |
| X   | <p>EUROPEAN JOURNAL OF IMMUNOLOGY<br/>vol. 20, no. 6, 1990, WEINHEIM DE<br/>pages 1265 - 1272<br/>Seman M.; Boudaly S.; Roger T.; Morisset<br/>J.; Pham G. 'Autoreactive T cells in<br/>normal mice: Unrestricted recognition of<br/>self peptides on dendritic cell I-A<br/>molecules by CD4-CD8- T cell receptor<br/>alpha/beta+ T cell clones expressing<br/>V(beta)8.1 gene segments'<br/>see the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: right;">-/--</p> | 8                                   |
| <p><sup>9</sup> Special categories of cited documents :<sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> |  |                                     |
| <b>IV. CERTIFICATION</b>  |  |                                     |
| <p>Date of the Actual Completion of the International Search</p> <p style="text-align: center;">22 JULY 1993</p>  | <p>Date of Mailing of this International Search Report</p> <p style="text-align: center;">JUL 22 1993</p>  |                                     |
| <p>International Searching Authority</p> <p style="text-align: center;">EUROPEAN PATENT OFFICE</p>  | <p>Signature of Authorized Officer</p> <p style="text-align: center;">GURDJIAN D.</p>  |                                     |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) |  |                       |
|--|--|-----------------------|
| Category °   | Citation of Document, with indication, where appropriate, of the relevant passages   | Relevant to Claim No. |
| X  | JOURNAL OF EXPERIMENTAL MEDICINE<br>vol. 175, no. 2, 1 February 1992, NEW YORK<br>US<br>pages 609 - 612<br>Nair S; Zhou F; Reddy R; Huang L; Rouse BT<br>'Soluble proteins delivered to dendritic<br>cells via pH-sensitive liposomes induce<br>primary cytotoxic T lymphocyte responses<br>in vitro.'   | 10                    |
| A  | see the whole document<br>---  | 18,19                 |
| X  | IMMUNOLOGY<br>vol. 74, no. 3, 1991, OXFORD GB<br>pages 399 - 406<br>MACATONIA S.E.; PATTERSON S.; KNIGHT S.C.<br>'Primary proliferative and cytotoxic<br>T-cell responses to HIV induced in vitro<br>by human dendritic cells'<br>see the whole document<br>---  | 11                    |
| X  | EMBASE DATABASE, Amsterdam, Ab.No.<br>91344512<br>Nishimura T. et al.<br>'Bispecific antibody-directed...'<br>& JPN. J. CANCER RES. (Japan) , 1991,<br>82/11 (1207-1210)<br>---  | 9                     |
| Y  | BLOOD<br>vol. 75, no. 12, 1990, NEW YORK<br>pages 2292 - 2298<br>CAUX C; SAELAND S; FAVRE C; DUVERT V; MANNONI<br>P; BANCHEREAU J 'TUMOR NECROSIS<br>FACTOR-ALPHA STRONGLY POTENTIATES<br>INTERLEUKIN-3 AND GRANULOCYTE-MACROPHAGE<br>COLONY-STIMULATING FACTOR-INDUCED<br>PROLIFERATION OF HUMAN CD34-POSITIVE<br>HEMATOPOIETIC PROGENITOR CELLS'<br>see the whole document | 1,3,4,6,<br>12        |
| A  | ---  | 2,5,7,<br>13,15       |
| Y  | JOURNAL OF INVESTIGATIVE DERMATOLOGY<br>vol. 97, no. 3, 1991, BALTIMORE US<br>pages 524 - 528<br>C.MOULON ET AL. 'A potential role for<br>Cd1a molecules...'<br>see the whole document<br>---  | 1,3,4,6,<br>12        |
|  | ---  | -/--                  |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) |   |                       |
|--|---|-----------------------|
| Category °   | Citation of Document, with indication, where appropriate, of the relevant passages  | Relevant to Claim No. |
| A  | <p>JOURNAL OF INVESTIGATIVE DERMATOLOGY<br/>vol. 94, no. 2, 1990, BALTIMORE US<br/>pages 166 - 173<br/>TEUNISSEN MB; WORMMEESTER J; KRIEG SR;<br/>PETERS PJ; VOGELS IM; KAPSENBERG ML; BOS<br/>JD 'Human epidermal Langerhans cells<br/>undergo profound morphologic and<br/>phenotypical changes during in vitro<br/>culture.'<br/>see the whole document</p> <p>---</p> | 1,3,12                |
| A  | <p>WO,A,9 106 630 (HAHNEMANN UNIVERSITY)<br/>16 May 1991<br/>see the whole document</p> <p>---</p>  | 1,3,4                 |
| A  | <p>WO,A,9 102 531 (PETER MACCALLUM CANCER<br/>INSTITUTE)<br/>7 March 1991<br/>see claim 15</p> <p>---</p>   | 1,2,4-7,<br>12,13     |
| A  | <p>EP,A,0 455 482 (BECTON DICKINSON &amp; CO.)<br/>6 November 1991<br/>see the whole document</p> <p>-----</p>  | 1-3,4                 |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9302639  
SA 72425

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
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| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)     | Publication<br>date  |
|---|---------------------|--------------------------------|----------------------|
| WO-A-9106630                              | 16-05-91            | US-A- 5128259<br>AU-A- 6722390 | 07-07-92<br>31-05-91 |
| WO-A-9102531                              | 07-03-91            | None                           |                      |
| EP-A-0455482                              | 06-11-91            | AU-A- 7429391<br>JP-A- 5076354 | 07-11-91<br>30-03-93 |